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Role of Allogenic NK Cells Treatment in the Early Phase of Apoptosis on Poorly Differentiated Retinoblastoma Cells Culture

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Abstract: **Introduction:** Retinoblastoma is a retinal malignant tumor because of mutations on chromosome 13q14. This is associated with apoptosis deregulation processes. Both autologous or allogeneic NK cells play their roles in both innate and adaptive immune systems. They can stimulate apoptosis through several mechanisms. **Methods:** This study determined the role of allogeneic NK cells in retinoblastoma cell apoptosis. Poorly differentiated retinoblastoma tissues were tested with SDS-PAGE. Allogeneic NK cells were isolated from peripheral blood of the healthy family members of patients. There were two culture groups consisting of retinoblastoma cells only as the control and retinoblastoma treated with allogeneic NK cells as the treated group. Each group consisted of 10 *well* plates. Examinations of the expression of Bcl-2, Caspase-3, and Apoptosis were performed by the flowcytometry method. **Result:** The SDS PAGE electrophoresis tests showed strong expression of proteins with molecular weights of 14, 19, 28, 35, 53 and 85 kDa. The percentage of early-phase apoptosis is higher than late phase. There were significant correlations ($\alpha < 0.05$) between Bcl-2, Caspase-3, and the ratio of both on cell apoptosis. **Conclusion:** In conclusion, allogeneic NK cells play a role in retinoblastoma cell apoptosis, especially in the early phase. It is expected to be one of the new strategies in cancer immunotherapy.

1 INTRODUCTION

Retinoblastoma is the most common eye cancer occurring in children. Retinoblastoma is a malignant tumor of the retina that is derived from primitive neuroectodermal tissues. This tumor is caused by mutations on chromosome 13q14. The most common occurrence is diagnosed when children are aged less than 3 years old. The incidence rate reaches approximately 4% of all malignancies in children. In the United States, retinoblastoma affects 1 in 18,000 children aged less than 5, and the prevalence reached 250 to 300 cases per year, resulting in a 1% death rate in all cases. The figures are higher in developing countries and continue to increase annually, making the success of retinoblastoma therapy a constant challenge for developing countries. There were 44 cases from 2010 to 2012 in Dr. Soetomo General Hospital in Surabaya (Bunin & Orjuela, 2007; Chantada & Leal-Leal, 2007; Chintagumpala *et al*., 2007; Soebagjo *et al*., 2013; Stewart & Wild, 2014).

Retinoblastoma is a tumor type that is known to be genetically influenced. The mutation of the tumor suppressor gene (RB1) which is located at chromosome 13q14 is closely related to tumor formation (Leiderman *et al*., 2007). Due to the genetic mutations occurring in retinoblastoma, proliferation will increase as apoptosis decreases. Homeostasis is achieved when the proliferation rate on the tissue is balanced with apoptosis. When apoptosis deregulation takes place, the number of dividing cells will be higher than the dying cells and in the end, a tumor is formed (Cheng *et al*., 2013).

The apoptosis process runs through two pathways, which are (1) the extrinsic pathway (cytoplasm) through the activity of the Fas death receptor by activating Fas ligand (FasL) interaction and (2) the intrinsic pathway (mitochondrial) that stimulates

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cytochrome-c release, which depends on Bcl-2 protein regulation (B cell lymphoma) as an anti-apoptotic protein and Bax as a pro-apoptotic protein. Singh *et al.* (2015) reported that the Bcl-2 is positively correlated to the growth of invasive retinoblastoma cells even though it is not correlated with Bax expression. However, Sitorus *et al.* (2009) reported that Bcl-2 overexpression would not prevent apoptosis, even though the independent Caspase-3 pathway is the apoptosis main pathway in retinoblastoma (Singh *et al.*, 2015; Sitorus *et al.*, 2009).

One of the cell types which can influence apoptosis occurrence is the Natural Killer (NK) cell. This cell constitutes lymphocytes playing a role in both the innate and adaptive immune systems. These cells can induce apoptosis through several mechanisms. In the last decade, the knowledge of NK cells, both the autologous and allogeneic, has developed and become one of the new strategies in cancer immunotherapy. Davis & Rizzieri (2015) mention that applying NK cells in atherapy on malignancy showed success and the studies in this field continue to grow rapidly. Eguizabal *et al.* (2014) state that NK cells play an important role in building immunity against cancer. Soebagjo *et al.* (2015) report that autologous NK cells play an important role in RB cells' aggressiveness through the expression of EZH2, Ki-67, and RB cell apoptosis (Davis & Rizzieri, 2015; Eguizabal *et al.*, 2014; Soebagjo *et al.*, 2015).

According to the facts, NK cell-based immunotherapy acts as one of the promising alternative therapies. So, investigations into the role of Bcl-2 protein as a regulator and Caspase-3 as the apoptosis executor on retinoblastoma cells treated with allogeneic NK cell will be the basic treatment especially as an alternative therapy for retinoblastoma cases.

2 METHODS

2.1 Research Subjects

Poorly differentiated retinoblastoma tumor tissues were collected from the enucleated patients of Dr. Soetomo General Hospital, Faculty of Medicine, University of Airlangga, Indonesia after obtaining informed consent. Fresh tumor tissues were tested using the SDS-PAGE method to determine the molecular weight of proteins. There were two groups consisting of retinoblastoma cells only as the control group and retinoblastoma versus allogeneic NK cells as the treated group. Each group consisted of 10 *well* plates of cells culture. Expression of Bcl-2, Caspase-3, and Apoptosis was examined with the flowcytometry test.

2.2 SDS-PAGE

The SDS-PAGE test is useful to describe the molecular weight of tissue. Basically, proteins in the tissue are extracted in a buffer gel. The gel is then divided into two parts, the stacking gel and separating gel. The stacking gel consisted of 830 µL of UGB (upper gel buffer), 534 µL of acrylamide (T-acryl), 1950 µL of ddH_2O, 40µL of ammonium persulfate (APS), and 4µL of tetramethylethylene diamine (TEMED). The separating gel consisted of 2600 µL of LGB (lower gel buffer), 4000 µL of acrylamide (T-acryl), 3400 µL of ddH_2O, 140 µL of ammonium persulfate (APS), and 14 µL of tetramethylethylene diamine (TEMED). As much as 3 µL of tumor tissue was prepared with µLTris-cl + 15 µL of RSB (Reducing Sample Buffer) and then it was separated by electrophoresis gelusing with 100 V voltage. The gel was then dyed with Coomassie Brilliant Blue R-250 so that the strands of molecules became visible. The molecular weight of each strand could be measured with a standard marker strand. The weight calculation of protein molecules was conducted based on Rf (Retardation factor) strandvalue of each sample. The migration distance of the polypeptide is a proportional inverse of the logarithm (log) value of the polypeptide molecule weight.

$$Rf = \frac{\text{Migration distance}}{\text{Gel length}} \quad (1)$$

2.3 Culture of Retinoblastoma Cells

The retinoblastoma tissue was cleaned 3 times with sterile PBS. It was then finely chopped in serum-free media of type-I collagenase. Finally, it was incubated for 30 minutes at 37ºC. After that, medium plus serum was added and then the tissue was filtered. Next, the tissue was put in a centrifuge for 10 minutes at the speed of 1600 rpm. The supernatant was discarded and the pellets were resuspended with medium plus serum. After that, the cell culture was conducted on plates so it became confluent. Multiple passages were conducted until cell lines formed. And then, the confluent culture was divided into two groups. The first was the control

group which was not treated with allogeneic NK cells and the second was treated with NK cells with the ratio of 1:1 (Soebagjo *et al.,* 2015).

2.4 Peripheral Blood Collection for Allogeneic NK Cells

The isolation of peripheral blood mononuclear cells (PBMC) was based on Boyum's method (1968) with some modifications. Isolation of mononuclear cells is often used to analyze the cellular immune responses by reacting antibody with mononuclear cell-surface antigens (Bøyum, 1968; Rantam, 2003).

The peripheral blood collection for NK cell isolation was performed on healthy biological family members of the retinoblastoma patients. The blood samples were stored in a K_3EDTA vacuum tube for PBMC isolation. In addition, the collection of NK cells can also be conducted through stem cell growth by adding GM-CSF growth factor to the NK cell culture process (Bøyum, 1968).

2.5 Examination of the Number of NK Cells ($CD3^-CD56^+/CD16^{+)}$

The NK cell examination was performed using flowcytometry by means of BD FACS Calibur™, reagents of BD TriTEST™ CD3, Fluorescein isothiocyanate (FITC)/CD16 CD56 phycoerythrin (PE)/CD45 and peridinin-chlorophyll protein (PerCP), which are the pigment reagents of immunofluorescein to determine the number of NK cells ($CD3^-CD56^+/CD16^{+)}$ (Hu et al., 2012).

The NK cells expressing $CD3^-$, $CD56^+$, and $CD16^+$ would experience fluorescence in accordance with the area and then gating was performed on NK cells in the expression areas of $CD56^+/CD16^+$ and areas without the expression of CD3 ($CD3^-$). The number of NK cells (cells/µL) was obtained by calculating the ratio of cell event expressing $CD3^-CD56^+/CD16^+$ with fluorescent bead event; the number was previously discovered on the BD TruCOUNT tube (Hu *et al.,* 2012).

2.6 Examination of Cells Expressing Bcl-2, Caspase-3, and Apoptotic Cells

The examination of cells expressing Bcl-2, Caspase-3, and apoptotic cells was conducted using flowcytometry by means of BD FACS Calibur™, reagents of the primary antibody of Anti-Bcl-2 (100) FITC and FITC Active Caspase-3 Apoptosis, and FITC Annexin V Apoptosis Detection Kit II Cat. 556 570, fixation reagents of BD Cytofix/Cytoperm™, permeabilization reagents of BD FACS Permeabilizing Solution 2, washer reagents of BD Perm/Wash™ buffer, and lysis reagents of *BD FACS* Lysing Solution. Staining with Annexin V is typically used in conjunction with a vital dye such as propidium iodide (PI) for identification of early and late apoptotic cells. Meanwhile, the number of cells that expressed Bcl-2 and Caspase-3 (cells/µL) was obtained by calculating the ratio of cell-event expressing antibodies with fluorescent bead events that was previously discovered on the BD TruCOUNT tube.

2.7 Statistical Analysis

All of the results were collected in specific data collection sheets, grouped and presented in tabular forms and diagrams, and then analyzed by using SPSS 15.0 so as to analyze the amount of Bcl-2 and Caspase-3 and apoptosis between two groups using a T-Test and the Mann-Whitney Test. The correlation analysis between the variables was conducted by Spearmann's Rank Correlation Test. The p value was <0.05 which statistically indicated a significance.

3 RESULTS

3.1 SDS-PAGE

The expression of protein fractions from poorly differentiated retinoblastoma samples was suggested from some arising proteins with the molecular weight range of 14-85 kDa. The SDS-PAGE electrophoresis test showed a strong expression of the protein with the molecular weights of 14, 19, 26, 35, 53 and 85 kDa (Figure 1). Some protein fraction expressions are in accordance with the theory on the possibility of some proteins with molecular weights contained in the poorly differentiated retinoblastoma samples.

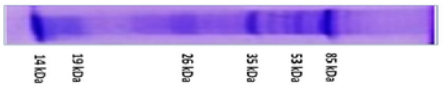

Figure 1: Profile of retinoblastoma protein with SDS-PAGE test. Protein expression in retinoblastoma samples including proteins with molecular weights of 14, 19, 26, 35, 40, 53, and 85 kDa.

The characterization overview of the culture of retinoblastoma cells showed cell growth between the control group and the treated group during the three

days' incubation period. The control group showed retinoblastoma cell growth shown by the grouping and confluent cell layers (Figure 2B).

In the treated group, there was less growth of retinoblastoma cells and the culture showed the formation of remnant sand of NK cells with the dead (apoptotic) cells surrounding between (Figure 2D).

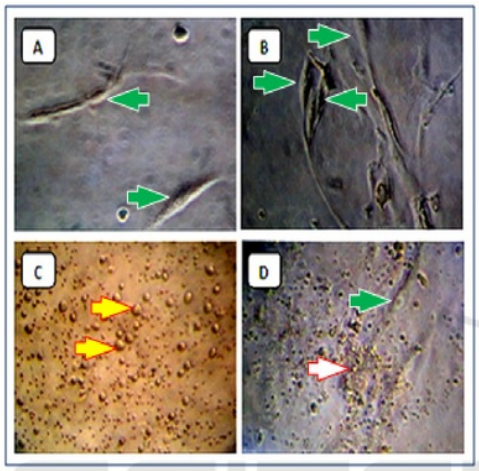

Figure 2. Pictures of culture retinoblastoma cells. A) Control group: culture of retinoblastoma cells (pre-seeding); B) Control group: culture of retinoblastoma cells 3 days post seeding; C) Treated group: culture of retinoblastoma cells with NK cells (pre seeding); D) Treated group: culture of retinoblastoma cells with NK cells 3 days post seeding; (Yellow arrow: NK cells; White arrow: overview of remnants of cell group undergoing apoptosis; Green arrow: intact retinoblastoma cells) (magnification: 400x).

4 DISCUSSIONS

4.1 Expression Percentage and Ratio of Bcl-2, Caspase-3, and Apoptosis on Retinoblastoma Cells Culture

The results showed the percentage of apoptotic cells, necrotic cells and living cells as well as the expression of antibodies Bcl-2 and Caspase-3 and the expression ratio of Bcl-2 and Caspase-3 in the control group and treated group. The expression was calculated based on the mean percentage (± SD) of positive cells expressing antibodies through the flowcytometry test.

The mean percentage of the cells that expressed Bcl-2 in the treated group was 0.10±0.10%, while in the control group the expression did not occur (0.10±0.10%). Caspase-3 expression in the treated group was 90.27±1.70%. It was higher compared with the control group (70.03±3.63%). The percentage of apoptotic cells in the treated group was 27.69% ± 2.36 . It was lower compared with the control group (37.72%±2:01).

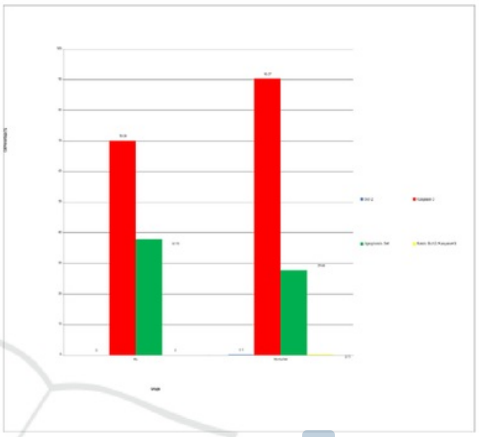

Figure 3: Percentage and ratio expression of Bcl-2, Caspase-3, and Apoptosis between the two groups.

4.2 The Effect of NK Cell Treatment on the Total Expression of Bcl-2, Caspase-3, and Cultured Retinoblastoma Cell Apoptosis

The effect of NK cell treatment on retinoblastoma cell apoptosis was calculated by the pigmentation of Annexin V/PI and flowcytometry analysis (Figure 4). The number of cells undergoing apoptosis was calculated based on the increasing concentration of apoptotic cells at early or late phases. Flowcytometry worksheets showed four areas to evaluate cell condition. Living cells can be observed on the lower left, early phase of apoptotic cells on the lower right, late phase on the upper right, and necrotic cells on the upper left side. As the marker of the apoptotic cells, it was strong positively with Annexin V and negative with PI.

The number of early apoptosis in the treated group was 20.17%±1.81, slightly higher than the control group (19.53±2.36). In contrast, the percentage of the late phase apoptosis in the treated group of 7.51±6.01 is significantly lower than the control group (18.2±1.09).

The ratio of Bcl-2 and Caspase-3 was calculated to describe the difference between the expression of each parameter. There was no difference between the two groups (treated group was 0.11 ± 0.11% versus control group of 0.00 ± 0.00%).

The different test result analysis of all variables using the Mann-Whitney test and Independent T-test between the treated group and control showed a significant result among them. In addition, Spearmann's Rank correlation test between variables and apoptosis showed a significant result

Table 1: Different Test Result Analysis Control and Treatment in Phase Apoptosis.

Apoptosis Phases	Groups	Mean	P
Early	Rb	19.53±2.36	0.505
	Rb +NK	20.17±1.81	
Late	Rb	18.2±1.09	0.000 *
	Rb+NK	7.51±6.01	

Note: * significant at α = 0:05

Table 2: Difference Test Results on all variables between the two groups.

Variables	Difference Test Results
Bcl-2	0.002*
Caspase-3	0.000*
Cell apoptosis	0.002*
The ratio of Bcl-2/Caspase-3	0.000*

Note: * significant at α = 0.05

Table 3: Correlation Test Result between Variables and Apoptosis.

Variables	Apoptosis	
	r_s	p
BCl-2	-0.605	0.005 *
Caspase-3	-0.657	0.002 *
The ratio of BCl-2/ Caspase-3	-0.609	0.004 *

Note: * significant at α = 0.05

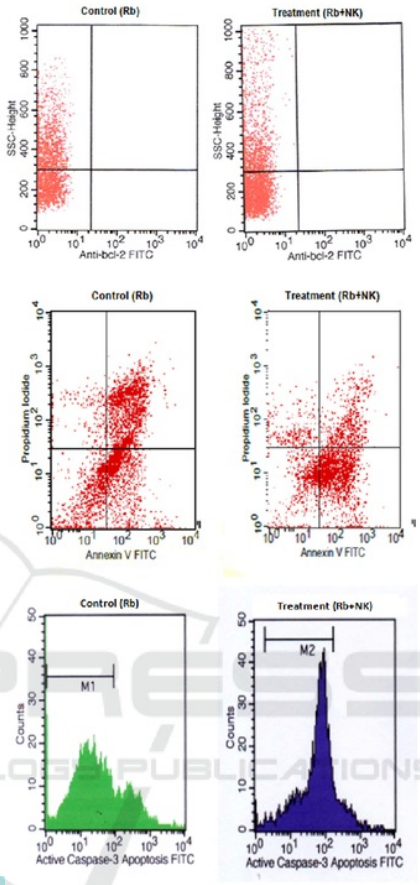

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Figure 4: Overview of flowcytometry on expression of Bcl-2, Caspase-3 and Apoptosis between the two groups.

A typical figure of retinoblastoma cell given NK cells is seen as the formation of remnant cells and NK cells surrounding cells that have an apoptotic process. Soebagjo *et al.* (2015) reported that NK cells have the ability to induce retinoblastoma cells thus inhibiting tumor cell growth and finally cause cell death. Therefore, NK cells are found surrounding the dead cells. In contrast, retinoblastoma cells grew normally in the control group (Soebagjo *et al.*, 2015).

The SDS-PAGE electrophoresis test on poorly differentiated retinoblastoma samples shows strong expression of proteins with the molecular weights of 14, 19, 26, 35, 53 and 85 kDa. The expressions show that there are proteins corresponding to Bcl-2 and Caspase-3 that play a role in retinoblastoma formation.

Raghupathi *et al.* (2003) measured the molecular weight of Bcl-2 (26 kDa) and Bax (21 kDa) by using immunoblot on HeLa cortex and hippocampus cells. In the cardiomyocytes, two types of Bcl-2 expressions with different molecular weights were obtained (26 and 32 kDa) (Raghupathi *et al.,* 2003; Cook *et al.,* 1999).

Caspase-3 with a molecular weight of 35 kDa is a form of fraction activated by T cells and is found in the cell cytoplasm. In addition, 35 kDa molecular weight is similar to the molecular weight of pro-caspase-7 and pro-caspase-9 (Paulse *et al.,* 2008; Dean *et al.,* 2002; Kadirvel *et al.,* 2010).

The process of cell death or apoptosis is commonly used as the selective parameter of a proposed anti-cancer source. Apoptosis induction was indicated by an increase in the percentage of cells undergoing apoptosis. The average of early apoptosis in the treated group was higher compared with the control group, while late apoptosis in the treated group was lower compared with the control group. So, NK cells play a role in early phase apoptosis. Cheng *et al.* (2013) mentioned the role of NK cells especially in the early stage of a good immune system both on innate and adaptive immune systems. Poggi *et al.* (2005) mentioned that the apoptosis trigger induced by NK cells will bind phosphatidylserine exposed on the surface of apoptotic cells and is marked by Annexin V in the early apoptosis phase. In contrast, in late-phase apoptosis, the death rate of both retinoblastoma cells and NK cells cannot be distinguished. Poggi *et al.* (2005) mentioned that the role of NK cells is effective on 24-hour incubation and it will reach the peak at 48 hours of incubation together inside tumor cells (coculture)(Cheng *et al.,* 2013; Poggi *et al.,* 2005).

The flowcytometry analysis showed that Bcl-2 is relatively insignificant in the apoptosis process. However, caspase-3 showed significant cell expression but apoptotic cells were lower compared with the control group. The correlation of Bcl-2/Caspase-3 showed that Bcl-2 yielded less expression when compared with Caspase-3. Nevertheless, Caspase-3 turned out to be inversely proportional to the occurring cell apoptosis. This shows the apoptotic signaling process that occurred in allogeneic NK cells in retinoblastoma cells. In general, the process of NK cell apoptosis goes through two pathways, which are (1) the intrinsic pathway which goes through the NKG2D ligand or (2) the extrinsic pathway which goes through the TNF ligand/FasL (Dranoff, 2004; Krzewki & Coligan, 2012).

The NK cells will induce tumor cells through Killer Activation Receptors (KARs), i.e. the NKG2D ligand when the KIR inhibitory signal is hampered because the tumor cells are inhibited by the expression of MHC class 1 which cause cytotoxic granules (perforin and granzyme) to not be excreted. The cytotoxic granules will be excreted if NK cell receptors, FcɣRIII (CD16) bind IgG tumor cells. Excreted perforin will open pores as the entrance of granzyme protein to mediate apoptosis (Dranoff, 2014; Abe *et al.,* 2008).

There are 5 types of granzyme known to exist in humans. These are granzyme A, B, H, K, and M. The difference of the types lies in the structure of the serine protease substrate (Grossman *et al.,* 2004). Hochegger *et al.* (2004) mention that the class of polymorphonuclear cells (PMN) excrete perforin and 2 types of granzyme which are granzyme A and B which contribute to the process of cytotoxic cells. Granzyme A induces cell death through the DNA characteristic of single chain cells and do not activate caspase cascade (Bots & Medema, 2006).

In NK cells, granzyme B plays a role. Granzyme B is capable of directly inducing apoptosis (bypass) via Caspase-3. This is consistent with the results of research and can occur because Bcl-2 is not sufficient to affect the apoptosis process. Overexpression of Bcl-2 in retinoblastoma itself is noted to not necessarily hinder the apoptosis process. The reason why Bcl-2 is not functional is supported by Sutton *et al.* (1997) who state that constraints on Bcl-2 will have an effect on increasing the ratio of the target and increase granzyme B. Granzyme B itself activates Bid pro-apoptotic proteins, along with Bax, which translocates to the mitochondria and affects the membrane permeability to release cytochrome-c after forming apoptosome by the Caspase cascade (Sitorus *et al.,* 2009; Bots & Medema, 2006; Sutton *et al.,* 1997; Pinkoski *et al.,* 2001).

In addition, Pinkoski *et al.* (2001) mentioned that in a direct path from granzyme B to caspase-3, apoptosis via the mitochondria can also occur via Smac/Diablo proteins that mediate apoptosis of granzyme B contributing to cell destruction by binding with XIAP. In addition, the release of Smac/Diablo in mitochondria will inhibit the Bcl-2 (Pinkoski et al., 2001; Verhagen et al., 2000; Adrain et al., 2001).

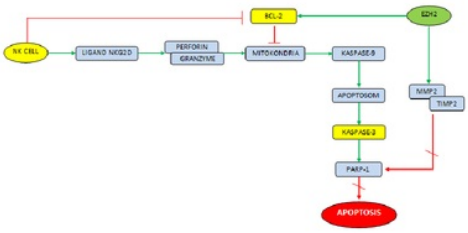

Figure 5: A schematic possible pathway of the induction of NK Cells on apoptosis in Retinoblastoma Cells.

The test results indicate that the ratio of the expression of Caspase-3 increased, reversely proportional to cell apoptosis. Sitorus et al. (2009) stated that there is a possibility that apoptosis occurs through Caspase-3 -dependent or independent pathways. Apoptotic signals that have come to the Caspase-3 effector are possibly hindered by DNA enzyme of repair poly(ADP-ribose) polymerase-1 (PARP-1). Active Caspase-3 protein will divide various substrates, including PARP-1 which is the mitotic apparatus core, lamina nucleus, and also actin and endonucleases. PARP-1 plays an important role in DNA repair processes by participating in the initiation of base excision repair (BER), nucleotide excision repair, and base repair with single-stranded DNA ligase III mediation, as well as controlling 60-70% of cell metabolism, cell cycle and cell transcription. Cell death signals of Caspase-3 towards PARP-1 cleave and the PARP-1 fragments will activate cell apoptosis. The cleavage process of PARP-1 is conducted by cytokine of Matrix Metalloproteinase-2 (MMP-2). However, the cleavage process by MMP-2 is inhibited by Enhancer Zeste Homolog-2 (EZH2) that represses micro RNA 21 promoters and inhibits MMP-2 through TIMP-2 inhibitors. Barriers on PARP-1 will result in the increased expression of Caspase-3 and in opposite proportion to cell apoptosis (Lazebnik et al., 1994; Nicolescu et al., 2009).

5 CONCLUSIONS

It is concluded that the provision of allogeneic NK cells play a significant role in the apoptosis process of poorly differentiated retinoblastoma cells, especially at the early phase through the intrinsic pathway.

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